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A simple fermentative process for ensuring safety and nutrition of legume and legume wheat based sourdoughs

V. Batra[1], M. Ghosh[2] and A. Ganguli[3*]

[1]Food Innovation Unit, S.R Enterprises, Baddi, Himachal Pradesh, India; [2]Department of Biotechnology, Thapar Institute of Engineering & Technology, Patiala, India; [3]SSD Projects Conglomerate, PHFI, Gurgaon, India; ganguliabhijit@rediffmail.com

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Abstract

The present study investigated the possibility of reducing non-nutritional factors in the widely cultivated legume *Vigna mungo*. This legume is widely cultivated and serves as an economical and important source of nutrition, lack of consumer friendly, adaptable methods in reducing the non-nutritional factors in the pulse, however, has been an impediment in the consumption (discomfort and other problems) of this legume. The application of lactic acid bacteria for reducing non-nutrients could be an interesting, effective and adaptable approach for legumes. In view of this, a simple fermentative method was designed in this study, for reducing the non-nutrients: trypsin inhibitor, cyanide, saponin, raffinose series oligosaccharides, tannin and phytate prevalent in *V. mungo.* Lactic acid fermentation carried out using an indigenous strain of *Lactococcus lactis* in *V. mungo* and cereal (wheat) composite flour led to the reduction of phytate and saponin by 69 and 81% after 4 h and complete removal after 8 h. Hydrogen cyanide, tannin and amylase inhibitor were also degraded completely; trypsin inhibitor decreased by 41% within 4 h and bound fructose (raffinose series oligosaccharides) in the *V. mungo* wheat sourdough was reduced by 65% after 4 h. No significant (P>0.05) change in the levels of total sugars, total proteins and lipid and polyphenols was observed. Moreover, the cell viability and production of gamma aminobutryic acid by *L. lactis* in the composite sourdough remained unaltered over the period of fermentation. The results of this study suggest an exciting possibility of exploiting the indigenous probiotic *L. lactis* strain for designing nutritional foods with *V. mungo*.

Keywords: anti nutrients, saponin, *Vigna mungo*, *Lactobacillus lactis*

1. Introduction

Black gram (*Vigna mungo*) is mainly a staple food, the dehulled and split seeds are a common dish in South Asia. The by-product of its processing (bran) constitutes about 15-20% of the seed weight and comprises hulls, germs and broken seeds. The bran is a potential feed resource and large quantities are available in India and other Southern Asian countries where black gram is a popular food. Besides being an important source of protein and carbohydrate, recent scientific evidence indicates several health beneficial effects for preventive chronic disorders in black gram. For instance, animal studies have revealed that polysaccharide and protein fractions have hypolipidemic action; glycosaminoglycan metabolism is altered upon inclusion of these fractions in the diet. Moreover neutral detergent fibres of black gram possess significant hypolipidemic and hypoglycaemic effects as well as anti-colon cancer activity (Apata, 2008). However, thus far wide acceptability of this popular legume is adversely affected due to the presence of tannins, phytic acid, protease, amylase inhibitors and about 76% of oligosaccharides of the raffinose family (Gemede *et al.,* 2014). These non-nutrients are responsible for reducing the availability of the nutrients in the diet through a diverse interplay of mechanisms. Complete removal of non-nutrients by breeding or other biotechnological methods, even if possible is not practicable since it would result in plants being characterised by poor growth and lower yields. Attempts to reduce the levels of the non-nutrients for improving the nutritional value of this food legume have largely resorted to soaking or germination of food legumes before the cooking process (Bhanwar *et al.,*

2013). However, most of the macro and micro nutrients, particularly vitamins and minerals as well as protein content are also lost during soaking and cooking (Bernfeld, 1955 and Baccou *et al.,* 1977). During the past decades, technological progress with lactic acid bacteria has been harnessed for improving products so that they are better suited to digestive capabilities and thus improve nutrition. Utilisation of lactic acid bacteria strains that perform both fermentation and remove non-nutrients is economically attractive and has been intensely investigated. Clearly, these achievements could be important for designing applications in the development of new food products with enhanced nutritional value. Earlier we reported the formulation of composite dough using *V. mungo* flour and wheat and the ability of a probiotic strain of *Lactococcus lactis* as a starter culture for preparing sourdough (Chavez-Gonzalez *et al.* 2011). The sourdough possessed significantly reduced tannin levels, besides other desirable technological properties for preparation of sourdough bread and cookies. We concluded that a systematic study to understand the fate of non-nutrients additionally present in *V. mungo* would be of considerable importance in designing an economically attractive alternative for nutritional enhancement by enabling more use of this underutilised, important legume by maximising the benefit-to-risk ratio.

In the present study, we report the fate of the non-nutrients trypsin and amylase inhibitors, saponin, phytic acid, cyanides, tannin and oligosaccharides of raffinose series, following fermentation of the dough by *L. lactis*. It may be envisaged that microbial degradation of non-nutrients in *V. mungo* may eventually offer valuable insights present for designing/developing food (s) with nutritional sustainability and utilisation of *V. mungo*.

2. Materials and methods

Microorganism and fermentation

The *L. lactis* subsp. *lactis* (Gene Bank accession number JN618456) previously characterised was routinely grown in De Man Rogosa and Sharpe agar (MRS) at 37 °C; inoculums were prepared by overnight growth of the microorganism in MRS medium at 37 °C with shaking (120 rpm). For fermentation, 10 g of composite flour was suspended in 100 ml of sterile distilled water and inoculated with log 10^8 cfu/ml of overnight grown *L. lactis* strain. The fermentation was allowed to proceed for up to 10 h at 37 °C. Samples were withdrawn periodically for analysis. In order to determine phytase activity, parallel sets lacking inoculum were used as controls. Cell viability was determined previously by the method as described by Bhanwar *et al.* (2013).

Determination of trypsin and amylase inhibitor activities of sourdough.

About 100 g sourdough sample (dried) was homogenised with 0.01 M phosphate buffer (pH 7.5) containing 0.1 M NaCl and analysed for trypsin inhibitor activity by the method as described by Hajela *et al.* (1999) and Campos-Vega *et al.* (2010). One unit of inhibitory activity is defined as the amount of inhibitor that suppressed 50% of proteolytic activity at 37 °C. For analysing amylase inhibitor activity, sourdough extract was incubated with amylase in presence of phosphate buffer (0.01 M) for 1 h at 37 °C. Control without enzyme was run simultaneously. The enzyme reaction was determined by the method as described by Bernfeld (1955) and Ejigui *et al.* (2005).

One unit of inhibitory activity is defined as the amount of inhibitor that reduced the amylase activity by one unit.

Saponin, phytic acid and tannin analysis

Saponin content was estimated from the methanolic extract by the method as described by Baccou *et al.* (1977) and Girigowda *et al.* (2006). Phytic acid was estimated from dried (powdered) fermented dough by the method suggested by Hajela *et al.* (1999). For estimating organic phosphorus, samples were treated with concentrated HCl and perchloric acid and analysed by the APHA method (Indira and Kurup, 2003). The AOAC (1980) protocol was used for determining tannin concentration. Folin-Denis reagent and saturated sodium carbonate were prepared freshly prior to analysis. Standard solution of tannic acid was freshly prepared by dissolving 10 mg of tannic acid in 100 ml water. Tannic acid (Sigma, St. Louis, MO, USA) standards were prepared in the range of 0-2.5 ml, aliquots in 25 ml volumetric flasks, then added to 1.25 ml Folin-Denis reagent and 2.5 ml sodium carbonate solution. The mixture was made up to the volume and the colour was measured after 30 min at 760 nm using a spectrophotometer (Shimadzu). The fermented and non-fermented samples (1 g) were boiled in 80 ml of water for 30 min. The samples were cooled, transferred into a 100 ml volumetric flask and diluted to mark. Tannin content was determined from tannic acid standard curve and expressed as milligrams of tannic acid equivalence per 100 g of flour sample. Total cyanide contents were estimated by the method as described by Chaouali *et al.* (2013). Briefly, twenty grams of the fermented and non-fermented *V. mungo* flour were weighed accurately and placed in a 1000 ml round-bottomed flask with 50 ml of distilled water and 10 ml of sodium acetate (0.02 N). Complete conversion of cyanogenic glycosides to hydrocyanic acid was carried out by incubation for 12 h at 38±2 °C and followed by steam distillation. The vapours of HCN were collected in a flask cooled in ice. About 100 ml of liquid so obtained was trapped in 50 ml silver nitrate and 1 ml of nitric acid (0.02 N), immediately transferred into a

graduated flask (500 ml) and diluted with double distilled water. The mixture was filtered and 250 ml of filtrate was collected, 2 ml of colour indicator added and excess silver nitrate titrated against a solution of ammonium thiocyanate (0.02 N) until appearance of brown coloured precipitate. Blank was run parallel and hydrocyanic acid was expressed as mg/kg and calculated using the formula below:

$$HCN = 0.54\ (V2\text{-}V1) \times \frac{500}{250} \times \frac{1000}{M}$$

Where, V1 = volume of ammonium thiocyanate required to neutralise excess silver nitrate in the sample; V2 = volume of ammonium thiocyanate required to neutralise excess silver nitrate in the blank; M = weight of sample.

Determination of sugar

Free sugars were extracted from the dried, powdered sourdough samples by the method as described by Kaur *et al.* (2000). Total soluble sugars were estimated from the pooled extract. For analysing total bound fructose of sucrose and raffinose series oligosaccharides, the free fructose was destroyed with 30% NaOH by the resorcinol-HCl method (Kim *et al.,* 2012) the sugar free residue was dried at 60 °C and was estimated for starch by the method as described by Yoshida *et al.* (1976) and Kobawila *et al.* (2005).

Total polyphenols content

For determining total polyphenols content (TPC), the spectrophotometric method as described by Singleton and Rossi (1965) and Khokar and Owusu Apenten (2011) was used. Briefly, 200 mg of the ground sample (dry weight basis) was extracted with 4 ml of acidified methanol for 2 h, centrifuged for 10 min. Then 1.5 ml of ten-fold diluted Folin and Ciocalten phenol reagent (Sigma) was added to the supernatant, followed by addition of 0.6% sodium carbonate solution. The samples were incubated at room temperature for 90 min and then absorbance read at 725 nm. The total phenolic content was calculated using a standard plot of gallic acid and expressed as mg gallic acid/g composite flour (dry weight basis).

Statistical analysis

All data are expressed as mean value ± standard deviation (SD) of three independent determinations. One way ANOVA and Duncan's multiple range tests were used to compare means and significance value.

3. Results and discussion

Non-nutritional components are chemical compounds synthesised in natural food and or feedstuffs during normal metabolism by various mechanisms (for example inactivation of some nutrients, diminution of the digestive process or metabolic utilisation of food/feed) but exert effects contrary to optimum nutrition (Soetan and Oyewole, 2009). Non-nutritional factors have beneficial effects on gastrointestinal tract in modifying the microflora count of the intestine and by promoting the growth of beneficial bacteria. However, high levels of non-nutritional factors can cause detrimental effects to humans and animal growth and performance by impairing intake, uptake or utilisation of other food and feed components or by causing discomfort, flatulence and stress to humans and animals. The structure of these non-nutritional factors and their chemical properties especially heat lability, dictate the choice of physical process that will be more effective in their reduction or removal, thereby minimising adverse biological effects (Shahidi, 1997). Non-nutritional factors mainly occur in pulses and grain legumes and foods and feed material prepared from grain legumes and pulses (Friedman, 2001) and is an important factor for lower consumption of *V. mungo*. Therefore in the present study, fermentation was envisaged as a simple process for investigating the fate of the non-nutrients in *V. mungo* flour (Figure 1). Sourdough constitutes an ecological niche that is highly suitable for lactic acid bacteria, the *L. lactis* subsp. *lactis* is occasionally found among the sourdough microbiota used in traditional Italian, Portuguese, French and Mexican bread-making, bringing about many beneficial functional changes. Bhanwar *et al.* (2013) proposed a composite sourdough using *V. mungo* flour and wheat, as an economical source of nutrition as well as utilisation of *V. mungo*. The suitability of an indigenous *L. lactis* isolate with probiotic properties for survival and fermentation of the composite dough and production of tannase by this strain were important leads

Figure 1. The *Vigna mungo* L (Hepper) is widely consumed and has been used in this study.

for investigating the fate of other important non-nutrients in *V. mungo*. Cell viability was not affected for up to 10 h at 30 °C, indicating excellent survival characteristics of the strain (Figure 2). The fermentation process caused no changes in protein and lipid contents; however a slight decline in carbohydrate content was noted (Table 1). The effects of the *L. lactis* fermentation process on trypsin inhibitory activity, amylase inhibitor activity and hydrogen cyanide are presented in Table 2. After 4 h fermentation, trypsin inhibitor activity was significantly (P<0.05) reduced. Although prolonged fermentation resulted in higher reduction; increased acidity and reduced acceptability was noted, therefore a 4 h fermentation time has been emphasised throughout our study. Similar studies have reported the reduction in trypsin inhibitors during natural lactic acid fermentation of cereals. Ejigui *et al.* (2005) and Kobawila *et al.* (2005) reported 41.7% reduction in trypsin inhibitor in yellow maize during 4 days fermentation while Osman *et al.* (2003) observed 37-58% decrease in three sorghum cultivars after 24 h fermentation. Decline in trypsin inhibitor levels have also been reported for corn and corn soybean blend (Osman, 2004) and in millet based gruel (Patterson *et al.,* 2017). Trypsin inhibitor is important for reducing protein digestibility, pancreatic hypertrophy and poor growth performance in rats, mice and chicks (Osman, 2004; Sharma and Kapoor, 1996; Patterson *et al.,* 2017). The reduction of trypsin inhibitor level therefore may be useful in improving nutritional quality of *V. mungo* with respect to protein digestibility. The *L. lactis* fermentation resulted in significant (P<0.05) decline in amylase inhibitor levels. Amylase inhibitor levels could not be detected after 4 h. Similarly, Sharma and Kapoor (1996) observed almost complete removal of amylase inhibitor during 48 h fermentation of pearl millet. Complete degradation of amylase inhibitor has also been noted in sorghum and yellow corn by the method suggested by Osman (2004), Ejigui *et al.* (2005) and Kobawila *et al.* (2005). Both trypsin and amylase inhibitor reduction may be attributed to the ability of *L. lactis* to hydrolyse these proteins. The ability of *Lactococcus* sp. to hydrolyse various proteins and peptides by lactococcal cell envelope associated proteinases have been reported (Reddy *et al.,* 2000). Reduction of amylase inhibitor has been attempted by invasive processes such

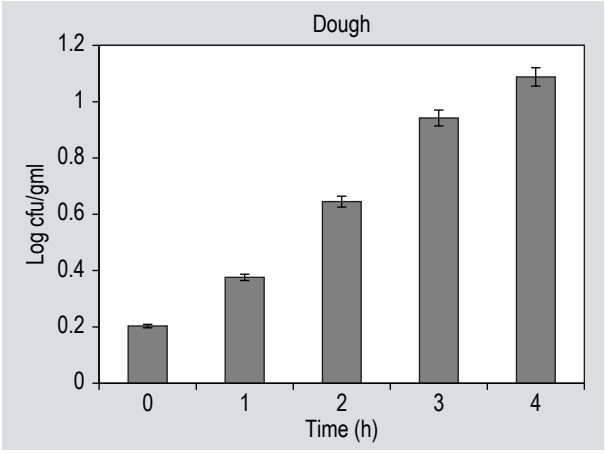

Figure 2. Cell count of *Lactococcus lactis* in *Vigna mungo* wheat sourdough fermented after 4 h. Values are mean of three replicates.

Table 1. Pattern of total sugar, starch, bound fructose, total protein and lipids in non-fermented and fermented *Vigna mungo* flour. Sugar concentrations are expressed as mg/g flour. Lipid concentrations are in %.

Sample type	Total sugars	Starch	Bound fructose (raffinose series oligosaccharides)	Total proteins	Total lipids (%)
V. mungo wheat dough – 0 h	50.46±4.21	408.22±11.0	10.66±1.60	198±3.7	1.2±0.05
V. mungo wheat-dough – 4 h	41.6±3.32	110±7.30	3.7±0.30	194±9.6	0.9±0.03

Table 2. Effect of *Lactobacillus lactis* fermentation on trypsin inhibitor activity, amylase inhibitor activity (IU/g flour) and hydrogen cyanide (mg/g) in wheat composite dough.[1]

Time (h)	Trypsin inhibitor activity	Amylase inhibitor activity	Hydrogen cyanide
0	96.8±8.10	3.68±0.60	34.6±3.21
2	72.4±4.08	1.1±0.02	4.1±0.12
4	56.3±2.20	ND	ND
6	38.0±0.41	ND	ND
8	18±0.22	ND	ND
10	4±0.03	ND	ND

[1] Data in parentheses indicate mean ± standard deviation; ND = not detected.

as heat treatment, germination, extrusion and radiation by the method as described by Osman (2004). However, a fermentative approach has not been reported for the legume *V. mungo* thus far and arguably stands out as the most favourable approach for removal of this non-nutrient. The reduction or elimination of amylase inhibitor may be useful for improving carbohydrate utilisation of *V. mungo*. Lactic acid bacteria especially *L. lactis* can tolerate and hydrolyse fairly high concentrations of cyanides in fermented cassava; possession of linamarase (β-glycosidase) is attributed to degradation of cyanides and cyanogenic glycosides during fermentation (Soris *et al*., 2010). The β-glycosidase production by *L. lactis* in composite sourdough has been noted in our earlier observations (results not shown). These results are in concurrence with other studies (Soris *et al.,* 2010) where *L. lactis* could degrade cyanogenic glycosides during fermentation. Phytic acid levels were reduced by 69.76% after 4 h, however a further decline was evident by 8 h. Though we observed phytase production by *L. lactis* strain in culture media, it is more likely that the lowering of pH, due to lactic acid production, may have been important in activating the legume intrinsic phytases resulting in reduction of phytic acid in the composite dough as proposed by Reale *et al.* (2007) and Sharma and Kapoor (1996).

A complete reduction of tannin (100%) following 4 h of fermentation was observed. (Khokar and Owusu Apenten, 2011; Sathe *et al.,* 1983) reported tannase activity of *L. lactis*; the extracellular tannase produced by this strain is thermostable and is not affected by the sourdough matrix (Figure 3). Tannase production by lactic acid bacteria has been an active research interest due to the importance of tannase in the food industry; *Lactobacillus plantarum* has been reported as an effective tannase producer (Sathe *et al.*, 1983). However the application of the strains used with the objective of reducing tannin in cereal/legume has not been reported.

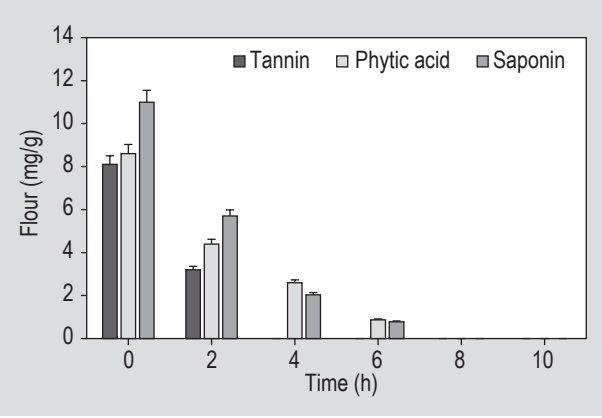

Figure 3. Levels of phytic acid, tannin and saponin of *Vigna mungo* wheat composite sourdough (mg/g flour) before and after fermentation. Results are mean ± standard deviation.

The concentration of saponins after 4 h of fermentation was reduced to 2.04 mg/g in the sourdough samples. Lower saponin content is desirable, as saponins retard growth and has been considered undesirable due to their toxicity and haemolytic activity (Singleton and Rossi, 1965). The role of Lactic acid bacteria in transforming saponins have been highlighted recently, for instance, ginsenoside, the ginseng saponin and principal bioactive component of ginseng was biotransformed by lactic acid bacteria isolated from kimchi (Park *et al.,* 2017). Though we presume a similar phenomenon in this case as well, detailed studies are mandatory for elucidating the mechanism. The oligosaccharides raffinose, stachyose, verbascose and ajugose constitute about 31-76% of the total soluble sugars (Yoshida *et al.,* 1976) in *V. mungo*. Due to the absence of α-galactosidase in humans these sugars are fermented anaerobically by microorganisms producing carbon dioxide, hydrogen and methane (Yoshida *et al.,* 1976) and are responsible for flatulence. In fact, high concentration of these sugars has been ascribed as one of the major reasons affecting the nutritional utilisation of *V. mungo*. As depicted, relatively low concentrations of sugars were detected in the sourdough samples after 4 h. Survival of microorganisms in sourdough matrix is likely to be achieved by metabolic adaptations; *L. lactis* is known to possess a strong capability for adaptation, moreover raffinose metabolism of *L. lactis* subsp *lactis (*strain A12*)* have been reported (Passerini *et al.,* 2010; Sharma and Kapoor, 1996).

The *L. lactis* strain used in our study is amylolytic and high α-amylase (36.9 U/ml) activity has been reported (Bhanwar *et al.,* 2013). This attribute, in addition to activated cereal and legume endogenous amylases during fermentation may have been responsible for the hydrolysis of starch. A comparison of the non-nutrient profile suggested notable decline of major non-nutrients in the fermented wheat-*V. mungo* dough, from a comparison with the available safety data of these non-nutrients, it may be inferred that consumption of the *L. lactis* fermented *Vigna* wheat flour is unlikely to pose health concerns (Table 3). In an earlier study, the probiotic, technological benefits of *L. lactis* and the consumer acceptability of wheat *Vigna* flour sourdough was demonstrated. The ability of *L. lactis* to produce gamma-amino butyric acid (GABA; 2.3 mg/g) was also reported by the authors in fermented *Vigna* wheat flour sourdough (Bhanwar *et al.,* 2013). The presence of GABA renders an additional therapeutic value to the sourdough and credence to the applicability of *L. lactis*.

The total phenolic content in fermented samples exhibited slight variations, a significant (P>0.05) change, however, was not noted (Table 4). Decrease of TPC content (and antioxidant activity) in cooked as well as in germinated *V. mungo* by the method as described by Gumbmann *et al.* (1989). Phenolic compounds are considered as an important source for beneficial physiological effects including antioxidant activities, amongst the legumes commonly

Table 3. Acceptable levels of antinutrients in *Lactococcus lactis* fermented *Vigna mungo* wheat dough.

Anti-nutrients	Residual level of antinutrient in fermented *V. mungo* wheat dough (g)	Toxicity levels
Alpha amylase inhibitor	Not detectable after 4 h	<3,000 mg/kg/kg body weight
Trypsin inhibitor	56.3 IU/g (approx. 18.66 mg/g) after 4 h	2.5 g/kg/kg body weight
Phytate	2.6 mg/g after 4 h	50-60 mg/kg/kg body weight
Tannin	Not detectable after 4 h	30 mg/kg/kg body weight
Saponin	2.04 mg/g after 4 h	0.2 g/kg/kg body weight
Hydrogen cyanide/cyanogenic glycosides	Not detectable after 4 h	50-60 mg/kg/kg body weight
Raffinose	3.7 mg/g	3.1 g/kg initiates 186/ml flatus volume causing discomfort in stomach

Table 4. Effect of fermentation on total phenolic contents in *Vigna mungo* wheat composite sourdough. Results are mean ± standard deviation.

Fermentation time (h)	Total phenolic content (mg/g)
0	5.61±1.11
2	5.22±2.21
4	4.91±2.01
6	4.62±1.19
8	4.33±2.13
10	4.10±3.01

consumed, black gram possesses the highest TPC. Our results suggest that fermentation does not lead to a substantial loss of phenolics therefore its contribution to the anti-oxidative activity may be presumed to remain unaltered. To the best of our knowledge, no studies, to date have attempted to explore a fermentative approach for reducing the non-nutrients in *V. mungo*. While the therapeutic and other benefits of the fermented *V. mungo* would be evident from clinical studies remains to be seen, the present study indicated a significant (P<0.05) reduction of major non-nutrients in the sourdough when *L. lactis* is used as a starter culture. It will be worthwhile to pursue the applicability of the strain for developing *V. mungo* conveniently for wider masses. We are currently elucidating the mechanisms for non-nutrient degradation of amylase and trypsin inhibitors by *L. lactis*.

4. Summary and conclusion

Overall, this study demonstrated the potential ability of an indigenous *L. lactis* culture to significantly (P<0.05) reduce the principal non-nutrients of *V. mungo* under ambient conditions. The non-nutrients amylase and trypsin inhibitors, hydrogen cyanide, phytate, tannin, raffinose series oligosaccharides and saponin were reduced in the laboratory formulated *V. mungo* wheat dough within a period of 4 h to residual levels notably below the established limits of toxicity and discomfort (resulting from flatulence). The total phenolic content, protein and lipids were not altered upon fermentation. The results of our study suggest a promising strategy to increase utility, safety and enhanced nutritional benefits of *V. mungo*. Besides, the production of GABA in the dough further imparts a therapeutic value. We are currently trying to understand the mechanism(s) of non-nutrient degradation of the indigenous *L. lactis* strain.

Conflict of interest

Authors have declared that they do not have any conflict of interest for publishing this research.

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